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الجامعة الأردنيـــة
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نموذج رقم (١٨)
اقرار والتزام بالمعايير الأخلاقية والأمانة العلمية
وقوانين الجامعة الأردنية وأنظمتها وتعليماتها
لطلبة الماجستير

أنا الطالب: سناء محسن موسى الرملاوي الرقم الجامعي: (٨٠٦١٧٢١)
تخصص: العلوم الطبية المخبرية الكليـــة: الطب

عنوان الرسالة:
Screening for Cystic fibrosis mutations among Jordanian patients

اعلن بأنني قد التزمت بقوانين الجامعة الأردنية وأنظمتها وتعليماتها وقراراتها السارية المفعول المتعلقة باعداد رسائل الماجستير عندما قمت شخصيا" باعداد رسالتي وذلك بما ينسجم مع الأمانة العلمية وكافة المعايير الأخلاقية المتعارف عليها في كتابة الرسائل العلمية. كما أنني أعلن بأن رسالتي هذه غير منقولة أو مستلة من رسائل أو كتب أو أبحاث أو أي منشورات علمية تم نشرها أو تخزينها في أي وسيلة اعلامية، وتأسيسا" على ما تقدم فانني أتحمل المسؤولية بأنواعها كافة فيما لو تبين غير ذلك بما فيه حق مجلس العمداء في الجامعة الأردنية بالغاء قرار منحي الدرجة العلمية التي حصلت عليها وسحب شهادة التخرج مني بعد صدورها دون أن يكون لي أي حق في التظلم أو الاعتراض أو الطعن بأي صورة كانت في القرار الصادر عن مجلس العمداء بهذا الصدد.

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تعتمد كلية الدراسات العليا
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التوقيع...... التاريخ...

SCREENING FOR CYSTIC FIBROSIS MUTATIONS AMONG JORDANIAN PATIENTS

By
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Dr. Mohammed S. El-Khateeb, Prof

Co-supervisor
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This Thesis is submitted as part of Fulfillment of the requirements for the Master's Degree of Science in Medical Laboratory Sciences.

Faculty of Graduate Studies
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May, 2011

COMMITTEE DICISION

This Thesis of (Screening for Cystic Fibrosis Mutations among Jordanian patients) was successfully defended and approved on Wednesday, May 11th, 2011.

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I dedicate this work to my family; my father and mother, my sisters and brothers for believing in me and supporting me all the time.

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LIST OF ABBREVIATIONS

ABC: ATP Binding cassette.
ASO: Allele Specific Oligonucleotide
Bp : base pair.

c-AMP: cyclic AMP.

CBAVD: Congenital Bilateral Absence of Van Deferens.

Cl: Chloride.

CF: Cystic Fibrosis.

CFTR gene: Cystic Fibrosis Transmembrane conductance Regulator gene.

CFS: Cystic Fibrosis Society.

DGGE: Denaturating Gradient Gel Electrophoresis.

DNA: Dioxyribo Nucleic Acid.

IVS8: Intron 8 splice variant.

MSD: Membrane Spanning Domain.

mRNA: messenger Ribo Nucleic Acid.

NCDEG: National Centre of Diabetes Endocrinology and Genetics.

NBD: Nucleotide Binding Domain.

PCR: Polymerase Chain Reaction.

PD measurement: Potential Difference measurement.

PS: primer Solution.

R Domain: Regulatory Domain.

RDB: Reverse Dot Blot.

RPM: Round per minute.

SSCP: Single-Stranded Conformation Polymorphism.

TBE: Tric Boric-Acid EDTA.

UV: Ultra- Violet Light.

µl: Micro Liter.

LIST OF APPENDICES

SCREENING FOR CYSTIC FIBROSIS MUTATIONS AMONG JORDANIAN PATIENTS

By
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ABSTRACT

Cystic fibrosis (CF) is the most common autosomal inherited recessive, life-limiting disease among the Caucasian population. It is a multisystem disease that results from loss of function in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene that located in chromosome 7. The main function of CFTR is to regulate the electrolyte movement across the epithelial cells. CFTR gene has more than 1500 known mutation and around 200 polymorphisms. The frequency of the mutations varies among different geographic and ethnic groups.

The aim of this study is to identify the common CFTR gene mutations (unidentified in Jordan previously), which would have important diagnostic implications.

Forty four unrelated cystic fibrosis Jordanian patients were included in this study. The patients were screened for the mutations in exons (2 ,3, 4, 7, 9, 10, 11 13,16,19,20 and 21) and introns (5, 12, 14b, 17a) using the 36 common mutations that available in a commercial INNO-LiPA CFTR 17+Tn and INNO-LiPA CFTR 19 Update diagnostic kits. Conventional PCR was used to detect the deletions in exon 2, 17-18. To detect the rest of unidentified mutations; sequencing was used as the last step in the mutation detection strategy.

Out of 44 unrelated patients (88 alleles) 8 mutations (Δ508F, N1303K, del. exon 2, del. exon 17-18, 1548delG, R117H, 2183AA->G and A559T) were identified, in 20.1% of patients. Two of these mutations (2183AA->G and A559T) were identified for the first time in Jordan. Only 4 out of 36 known mutations, using the commercial kit, were identified (11.1%) which makes it inadequate screening method for the detection of the mutations.

Δ508F represents (4.5%) in our study which considered low as reported in the previous studies conducted in Jordan. Two novel polymorphisms also detected through this study

(1538A>G exon 10 and 1744 A>C exon 11). Interestingly, no other polymorphisms were identified in this study.

In conclusion, further studies is needed to identify the mutations in other exons in order to investigate the common mutations in our population, in which can be used for diagnosis, screening program and prenatal diagnosis.

INTRODUCTION

1.1 CYSTIC FIBROSIS: DISEASE REVIEW

Cystic fibrosis (CF; MIM# 219700) also called mucoviscidosis is the most common, classic mendelian autosomal recessive, life-limiting disease among the Caucasian population. It occurs in about 1/2500 Caucasian births (Welsh, 1995). CF was believed to be rare among Arabs probably due to the lack of screening programs and careful surveys.

It affects the epithelia in several organs resulting in a complex, multisystem disease, includes the exocrine pancreas, intestine, respiratory tract, male genital tract, hepatobiliary system, and exocrine sweat glands. It is a multisystem disease that results from loss of function in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (CFTR; 602421) discovered in 1989 (Riordan, 1989, Rommens, 1989), encoding the c-AMP activated chloride channel, classically leading to respiratory tract, gastrointestinal, pancreatic, and reproductive abnormalities. A defective gene alters a protein that regulates the normal movement of salt (sodium chloride) in and out of cells which causes an increased concentration of sweat electrolytes. This lifespan illness gets more severe with age and can affect both males and females.

In recent decades, the lifespan of CF patients has been improved due to the early diagnosis (i.e., newborn screening tests), care (i.e., muconium ileus management, the methods for sputum clearance and the managing of respiratory failure) and disease therapy (i.e., improved antibiotics). Almost 80% of CF patients reach adulthood. (Welsh, 1995, Marie and Atul, 2005).

Since CFTR gene has more than 1500 mutations and more than 200 polymorphisms are described, the frequency of a certain mutations varies among different populations depending on geographical and ethnical nature. The majority of CFTR functional defects are the point mutations in which missense mutations represent (42%), small insertions/deletions with a frame shift (24%), nonsense mutations (16%), mutation of splicing (16%) and deletion of amino acids (2%).

The most frequent mutation is a result of a deletion of three nucleotides resulting in a deletion of phenylalanine at position 508 of CFTR gene that is responsible of 70% of CF alleles worldwide. But in Jordan, according to the study conducted by Dr. Rawashdeh and Haddad (2000), (Δ508F) mutation was reported only in (7.4%) among Jordanian patients.

1.2 AIMS AND OBJECTIVES

1. Identification of the common CFTR gene mutations, which would have important diagnostic implications.
2. To compare detected mutations with those reported worldwide in other populations.
3. To establish a diagnostic mutation screening profile suitable for Jordanian CF patients

LITERATURE REVIEW

2.1 CYSTIC FIBROSIS: DISEASE HISTORY

Cystic fibrosis (CF) was first documented as a specific medical condition during the 1930's. The early entries refer to the condition as '*cystic fibrosis of the pancreas'*. Interestingly, the condition was not part of the published 1032 pages on 'Common Disorders and Diseases of Childhood', published by Sir Frederick Still, in 1927. In 1936, Guido Fanconi established the relationship between celiac disease, bronchiectasis and cystic fibrosis. In 1938, the 'Cystic Fibrosis of the Pancreas and its Relation to Celiac Disease: a Clinical and Pathological Study' was published in the American Journal of Diseases of Children, describes the characteristics of the condition in detail. This publication by Dorothy Hansine Andersen described the onslaught of cystic fibrosis and correlated it with diseases of the lungs and intestines. She also hypothesized that the condition is recessive in nature. In 1988, Francis Collins, John R. Riordan and Lap-Chee Tsui discovered the first mutation for CF, ΔF508, located on chromosome 7 and the research further confirmed over a thousand different mutations that lead to the disorder. In 1989, Lap-Chee Tsui went on to probe the condition and discovered the gene responsible for cystic fibrosis at the Hospital for Sick Children, Toronto, Canada. Subsequent research on the disorder revealed that the microscopic mutations could only be mapped with the use of protein markers and gene-linkage. Thereafter, chromosome observation techniques such as 'walking' and 'jumping' were used to sequence the CF causing gene. In the Middle East a review was made by Dawson and Frossard in 2000 describing the history of CF.

The Table below shows the previous studies concerning the incidence of Cystic Fibrosis in many countries in the Middle East. Jordan has the highest rank with 1/2560 live birth (Nazar, 1992). The prevalence rate was estimated by meconium albumin method.

Despite the lowest incidence found in UAE and Bahrain, the authors reported that the spectrum of clinical presentation of the disease is severe in those countries.

The major outcome from these studies on the prevalence of CF is the lack of awareness, consanguineous marriage and poor diagnostic facilities. Warm climates, high humidity and insufficient salt intake (metabolic alkalosis) and prolonged neonatal jaundice are major factors that make the gulf region has CF in non-classical manner.

The first study performed in Jordan to detect the common CF mutations was carried by Dr. Mohammad Rawashdeh and Dr. Manal Haddad (2000). This study included 202 Jordanian and Palestinian patients. There were 114 (56%) boys and 88 (44%) girls. More than one CF case was found in 42 families out 120 families. Consanguineous marriage was present in 114 (69%) cases and parents were 1^{st}- 2^{nd} degree cousins in 86 (52%) of cases. Using a multiplex heteroduplex shift analysis followed by direct sequencing in 84 children with CF and 66 parents, 24 different CFTR mutations were identified. The DF508 mutation was found to account for only 7.4%, and six alleles were found for the first time (296+9A->T, T338M, T760M, 3670delA, 2006delA and G1244D).

Another study was performed by Abeer A.Freihat and Mohammed El-khateeb (May, 2007). This study included 54 unrelated patients, all of Jordanians & Palestinian origin. There were 20 (44%) males and 25 (56%) females. Using DGGE and SCCP along with

sequencing, the results were as follows: common mutations detected including: (ΔF508, N1303K, G85E, deletion Exon2, Del. Exon 17-18, 1548delGand Q1100P, TUB9 A>G, M470V), in addition to novel polymorphisms reported for the first time worldwide: (3272-92 T>C Heterozygous t>c (Intron 17a) and 1679G>T (10 exon))

Table 1.2: The important milestones in clinical and mutational findings in CF in the Middle East.

Country	Year	Authors	No. of patients	Incidence among live birth	Major CFTR mutations
Lebanon	1997	Marie Desgeorges	20	1 per 17,000	- 4010del4 - S4X - 4096-28G>A - E672del - M952I - ΔF508 - W1282X - N1303K - 2789+5G>A
Jordan	1984 1992 2000 2007	Kamal Nazar Rawashdeh Friehat.A	12 3 202 54	1 per 2560	- ΔF508 - N1303K - G85E - 1677del AT - 1548delG - Q1100P - Exon 2deletion - Del exons 17-18
UAE	1997	Frossard and Dawson	17	1per 15,000	- S549R - ΔF508
Kuwait	1986	Hans Kollberg	17	1 per 3,500	
Saudi Arabia	1998 1998 1998 1999	El-Harith Banjar et al. Banjar et al. Banjar et al	15 70 70 49	1per 4243	- 3120+1G→A - N1303K - 1548delG - ΔF508 - I1234V - N1303K - R553X - 3120+1G→A - H139L - S549R - 711+1G>T - L1177X - 425del42

1-2 CFTR GENE

1.2.1 CYSTIC FIBROSIS GENE AND ITS PROTIEN

The CFTR gene was discovered in 1989 (Riordan, 1989) when Riordan isolated overlapping cDNA clones from epithelial cell libraries with a genomic DNA segment containing a portion of the putative CF gene. Transcripts approximately 6,500 nucleotides in size were detectable in the tissues affected in patients with CF. The predicted protein consists of 2 similar motifs, each composed of a hydrophilic membrane-spanning domain (MSD) containing six helices and an important hydrophilic region for binding with ATP (NBF or Nucleotide Binding fold). These two motifs are linked by a cytoplasmic (R domain) encoded by exon 13, containing a number of charged residues and the majority of the phosphorylation sites (probable substrates of protein kinases A and/or C).

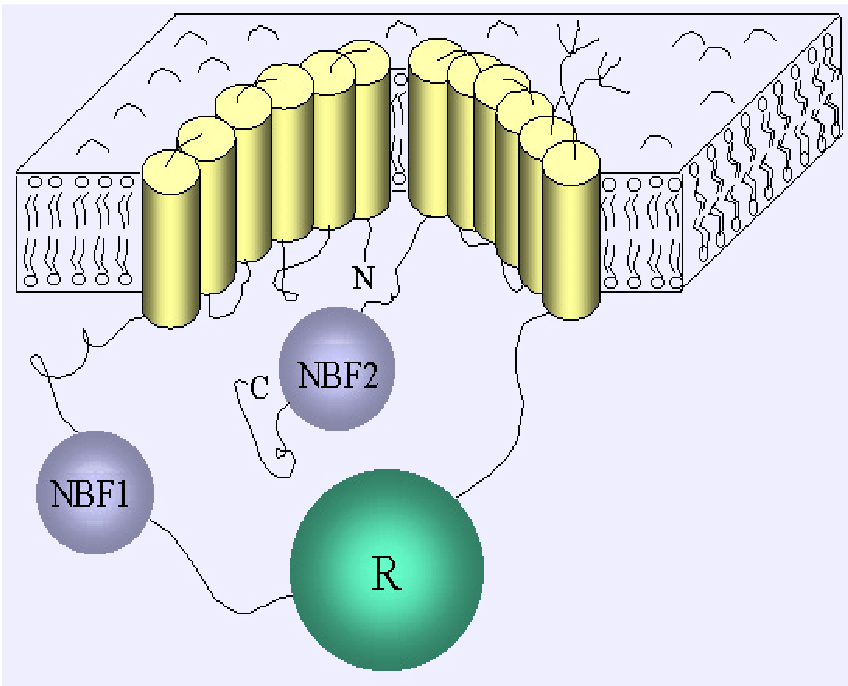


Figure1.1: proposed structure CFTR protein, by Riordan et al. 1989 - Pascale Fanen.

Each of these motifs has a domain property consistent with membrane-association and a domain believed to be involved in ATP binding. In CF patients, a deleted phenylalanine residue occurs at the center of the putative first nucleotide-binding fold (NBF). The predicted protein has 1,480 amino acids with a molecular mass of 168,138 Dalton.

CFTR located on the long (q) arm of chromosome 7 at position 31.2, is 230,000 base pair. CFTR gene contains 27 exons. The gene encodes a protein consisting of 1480 amino acids.

CFTR is a type of protein classified as an ABC (ATP-binding cassette) transporter or traffic ATPase. These proteins transport molecules such as sugars, peptides, inorganic phosphate, chloride, and metal cations across the cellular membrane. CFTR transports chloride ions (Cl^-) ions across the membranes of cells in the lungs, liver, pancreas, digestive tract, reproductive tract, and skin. (Baskin et al.).

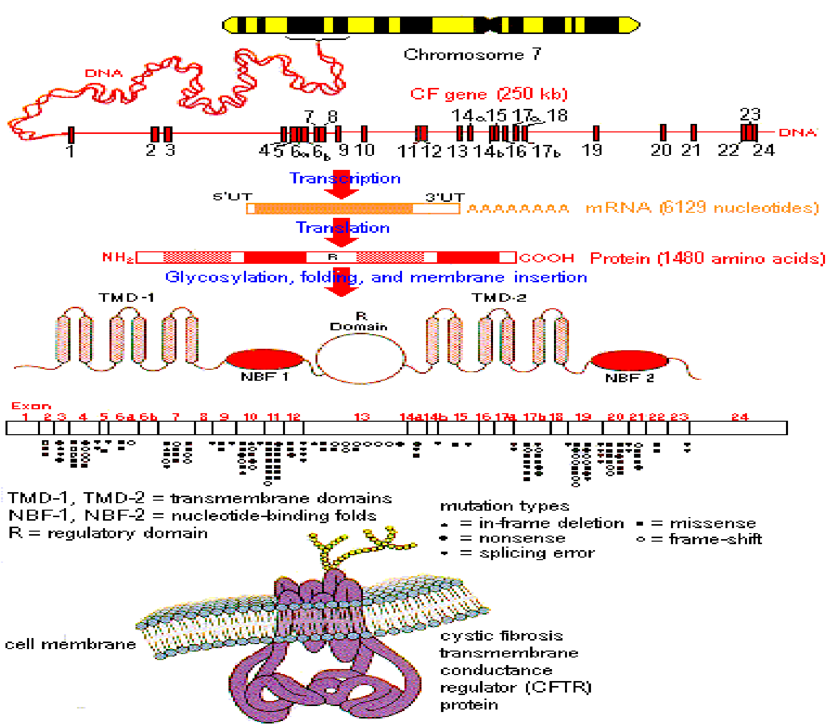


Figure 1.2: CFTR gene location and its protein. John R Riordan and Francis Collins (1989)

The structure of the complete CFTR protein has not yet been experimentally determined. This is because membrane proteins, such as CFTR, with substantial hydrophobic ("water-fearing") regions are extremely difficult to crystallize, and X-ray crystallography can only be carried out on protein crystals.

By comparing the CFTR protein sequence with that of other known ABC transporters, models depicting the structure of CFTR have been proposed (Figure 1.3). CFTR is made up of five domains: two membrane-spanning domains (MSD1 and MSD2) that form the chloride ion channel, two nucleotide-binding domains (NBD1 and NBD2) that bind and hydrolyze ATP (adenosine triphosphate), and a regulatory (R) domain. Delta F508, the most common CF-causing mutation, occurs in the DNA sequence that codes for the first nucleotide-binding domain (NBD1).

While most ABC transporters consist of four domains (two membrane-spanning and two nucleotide-binding domains), CFTR is the only one known to possess a regulatory domain.

Modification of the regulatory domain, either through the addition or removal of chemical phosphate groups, has been shown to regulate the movement of chloride ions across the membrane.

Although there are no structures of the entire CFTR protein in the Protein Data Bank (PDB), an international archive of molecular structure data, a structure for a similar ABC transporter is available from the PDB. In September 2001, the journal *Science* published an article on the X-ray structure of a CFTR-related ABC protein (Msba) in *E. coli*. The

PDB ID for this protein is IJSQ. PDB also contains a structure based on the theoretical model of the first nucleotide-binding domain (NBD1). PDB ID for this entry is 1NBD (Figure 1.3). In addition, PDB contains synthetic peptide structures (25 to 26 amino acids long) of NBD1's alpha helical region containing the delta F508 mutation. PDB IDs for structures of these peptides are 1CKW, 1CKX, 1CKY, and 1CKZ.

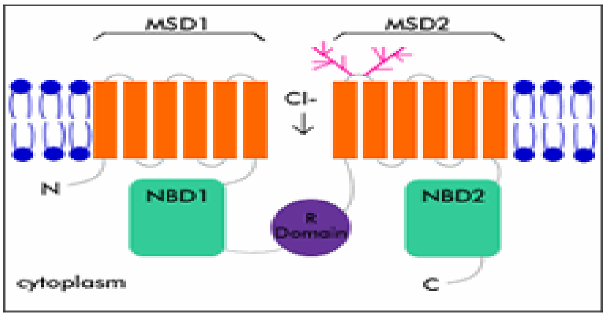


Figure1.3: Diagram depicting the five domains of the CFTR membrane protein (Sheppard 1999).

1.2.2 CYSTIC FIBROSIS: GENE MUTATIONS

About 70% of mutations observed in CF patients result from deletion of three base pairs in CFTR's nucleotide sequence. This deletion causes loss of the amino acid phenylalanine located at position 508 in the protein; therefore, this mutation is referred to as delta F508 or ΔF508 (Kerem ,1989)

With normal CFTR, once the protein is synthesized, it is transported to the endoplasmic reticulum (ER) and Golgi apparatus for additional processing before being integrated into the cell membrane. When a CFTR protein with the delta F508 mutation reaches the ER, the quality-control

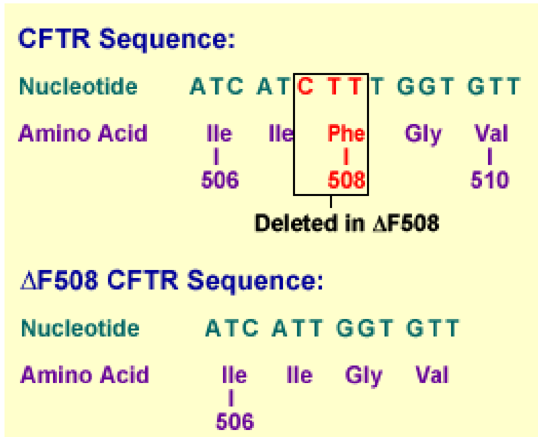


Figure 1.4: The deltaF508 deletion is the most common cause of cystic Fibrosis. The isoleucine (Ile) at amino acid position 507 remains unchanged because both ATC and ATT code for isoleucine

mechanism of this cellular component recognizes that the protein is folded incorrectly and marks the defective protein for degradation. As a result, delta F508 never reaches the cell membrane.

People who are homozygous for delta F508 mutation tend to have the most severe symptoms of cystic fibrosis due to critical loss of chloride ion transport. This upsets the sodium and chloride ion balance needed to maintain the normal, thin mucus layer that is easily removed by cilia lining the lungs and other organs. The sodium and chloride ion imbalance creates a thick, sticky mucus layer that cannot be removed by cilia and traps bacteria, resulting in chronic infections. While the mechanism that leads to lung damage is not fully understood, lung disease is the leading cause of morbidity and mortality among CF patients.

The molecular anomalies have variable effects on the CFTR protein and its functions.

Welsh and smith (1993) have proposed a classification of these anomalies in relation to the Cl^- channel function.

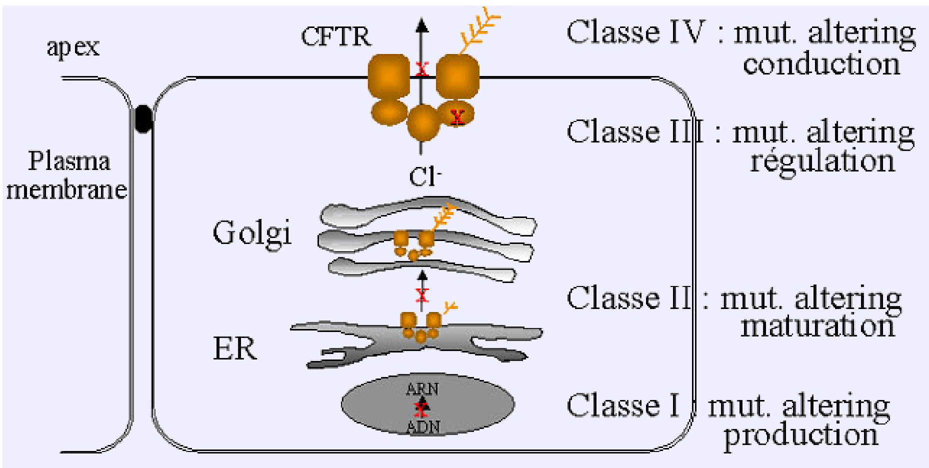


Figure1.5: Classification of mutations of CFTR gene, by Welsh & Smith, 1993 - Pascale Fanen

Class 1: mutations altering the production of the protein. These mutations result in the total or partial absence of the protein. This class includes the nonsense mutations and those that produce a premature stop codon (anomalies of splicing and frameshift mutations). In certain cases the mutated mRNA is unstable and does not produce the protein. In other cases, the abnormal protein produced will probably be unstable and degrade rapidly. This is what produces the truncated protein or the protein containing the aberrant sequence (anomalies of splicing or the frame shift). Functionally, these mutants are characterized by a loss of conductance of Cl- channel in the affected epithelia.

Class 2: mutations altering the cellular maturation of the protein. A number of mutations alter the maturation of the protein and thus the transport of these proteins to the plasma membrane. In this way, the protein is either absent from the plasma membrane or present in a very small quantity. The mutations of this class represent the majority of CF alleles (DF508).

Class 3: mutations disturbing the regulation of Cl- channel. These mutations are frequently situated in the ATP binding domain (NBF1 and 2).

Class 4: mutations altering the conduction of Cl- channel. Certain segments of membrane spanning domains participate in the formation of an ionic pore. The missense mutations situated in these regions produce a correctly positioned protein that has a cAMP dependant Cl- channel activity, but, the characteristic of these channels is different from those of endogenous CFTR channels with a diminution of ion flux and a modified selectivity.

Class 5: mutations altering the stability of mRNA.

Class 6: mutations altering the stability of mature CFTR protein.

1.2.3 CYSTIC FIBROSIS: INHERETANCE

Cystic fibrosis is inherited in an autosomal recessive pattern, which means both copies of the gene in each cell have mutations. The parents of an individual with an autosomal recessive condition each carry one copy of the mutated gene, but they typically do not show signs and symptoms of the condition.

An affected person usually has unaffected parents who each carries a single copy of the mutated gene (and are referred to as carriers). Two unaffected people who each carries one copy of the mutated gene have a (25%) chance with each pregnancy of having a child affected by the disorder, (50%) chance of having a child who has inherited one working gene copy and one mutated gene copy. They will be a carrier of the autosomal recessive disorder. Lastly, there is a (25%) chance of having a child who is completely unaffected by the autosomal recessive disorder.

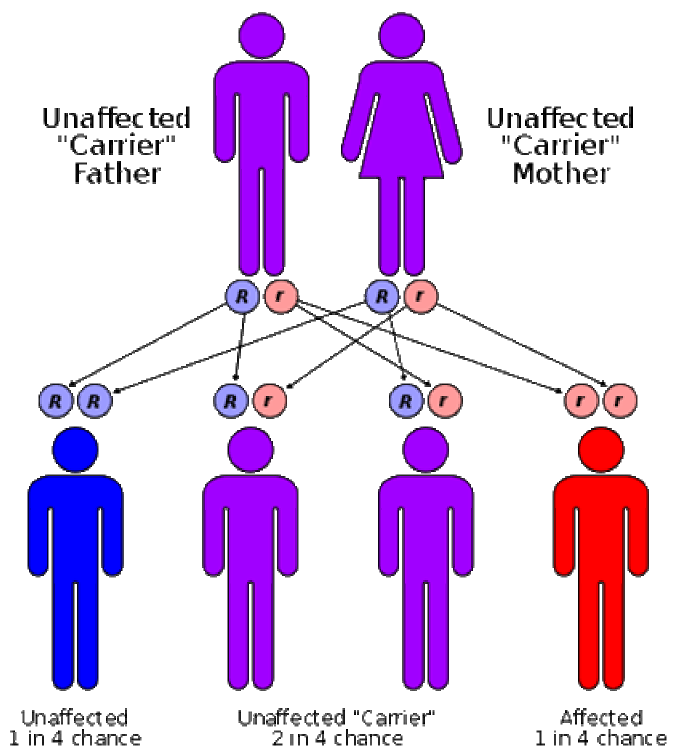


Figure 1.6: CF inheritance pattern (www.genet.sickkids.on.ca/cf/ inheritance)

The chance of being cystic fibrosis carrier will be increased according to the relatedness the family (brothers/sisters or cousins). About 80-90% of CF patients are born with predisposed mutant gene.

1.2.4 CYSTIC FIBROSIS: SYMPTOMS AND SIGNS

Symptoms of cystic fibrosis may vary from person to person and from time to time. Some people with this disease may experience severe lung and digestive problems. Other CF patients may observe severe symptoms till they reach adolescence. Most of the people suffering from this condition are likely to experience severe breathing problems, and respiratory failure is found to be the common reason for their death. One of the first signs of cystic fibrosis (CF) that parents may notice is that their baby's skin tastes salty when kissed or the baby does not pass stool when first born (Meconium ileus).

Most of the other signs and symptoms of CF develop later. They are related to how CF affects the respiratory, digestive, or reproductive systems of the body.

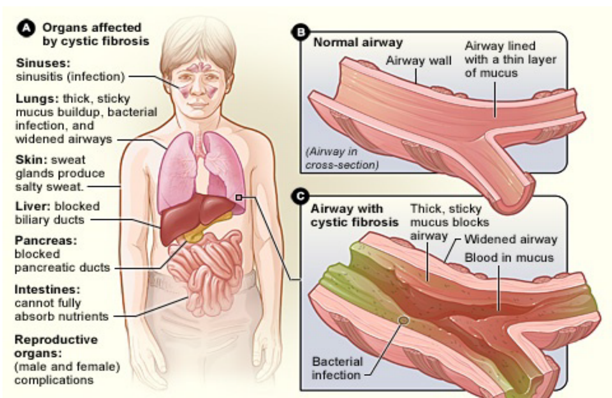


Figure1.7: A) shows the organs that cystic fibrosis can affect. B) Shows a cross-section of a normal airway, C) shows an airway affected by cystic fibrosis. The widened airway is blocked by thick, sticky mucus containing blood and bacteria. (www.genet.sickkids.on.ca)

I. Respiratory System Signs and Symptoms

People who have CF have thick, sticky mucus that builds up in their airways. This buildup of mucus makes it easier for bacteria to grow and cause infections. Infections can block the airways and cause frequent coughing that bring up thick sputum (spit) or mucus that is sometimes bloody.

People who have CF tend to have lung infections caused by unusual germs that do not respond to standard antibiotics. For example, lung infections due to bacteria called mucoid Pseudomonas are much more common in people who have CF. An infection caused by this bacteria may be a sign of CF.

People who have CF have frequent bouts of sinusitis, an infection of the air-filled spaces behind the eyes, nose, and forehead. Frequent bouts of bronchitis and pneumonia also occur. These infections can cause long-term lung damage.

As CF gets worse, patients may develop more serious complications, such as pneumothorax , or collapsed lung; or bronchiectasis . Some people who have CF also develop nasal polyps (growths in the nose) that may require surgery.

II. Digestive System Signs and Symptoms

Mucus blocks tubes, or ducts, in the pancreas and prevents enzymes from reaching the intestines causes most digestive system signs and symptoms.

Without these enzymes, the intestines cannot fully absorb fats and proteins. This can cause ongoing diarrhea or bulky, foul-smelling, greasy stools. Intestinal blockage also

may occur, especially in newborns. Too much gas or severe constipation in the intestines may cause stomach pain and discomfort.

A hallmark of CF in children is poor weight gain and growth. These children are unable to get enough nutrients from their food due to the lack of enzymes to help to absorb fats and proteins.

As CF gets worse, other complications may occur, such as:

- Pancreatitis.
- Rectal prolapse. Frequent coughing or problems passing stools may cause rectal tissue from inside to move out of the rectum.
- Liver disease due to inflamed or blocked bile ducts.
- Diabetes.
- Gallstones.

III. Reproductive System Signs and Symptoms

Men who have CF are infertile because they are born without a vas deferens. This is the tube that delivers sperm from the testicle to the penis.

A woman who has CF may have a hard time getting pregnant because of mucus blocking her cervix or by other CF complications.

IV. Other Signs, Symptoms, and Complications

Other signs and symptoms of CF are related to an upset of the balance of minerals in blood.

CF causes sweat to become very salty. As a result, the body loses large amounts of salt when it sweats. This can cause dehydration, increased heart rate, tiredness, weakness, decreased blood pressure, heat stroke, and, rarely, death.

CF also can cause clubbing nails and low bone density. It develops late in CF because the lungs are not moving enough oxygen into the bloodstream.

Osteoporosis also tends to occur late in CF.

Congenital Bilateral absence of Vas Deference (CBAVD)

Congenital bilateral absence of the vas deferens occurs in males when the tubes that carry sperm out of the testes (the vas deferens) fail to develop properly. Although the testes usually develop and function normally, sperm cannot be transported through the vas deferens to become part of semen. In 97%–98% of men with CF, a congenital bilateral absence of the vas deferens (CBAVD) blocks the transport of spermatozoa from testicular or epididymal structures to the outer genital tract, resulting in azoospermia (Lissens,1999, Chillon,1995).

Congenital bilateral absence of the vas deferens (CBAVD) is a genital form of cystic fibrosis (CF) that is responsible for 2%–6% of male infertility. The incidence of CF varies in different populations; therefore, the incidence of CBAVD will also vary in different populations. The spectrum and distribution of cystic fibrosis transmembrane conductance regulator (*CFTR*) gene mutations differ between CBAVD and CF patients and are comparable to control individuals. Combinations of particular alleles at several polymorphic loci yield insufficient functional CFTR protein. *CFTR* mutations are also

associated with congenital absence of the uterus and vagina (CAUV). Females with CF are found to be less fertile than normal healthy women. In CBAVD patients having 2 mutant *CFTR* alleles, at least one will be a mild mutation. In CBAVD patients in which a mutation is found on both *CFTR* genes, about 88% carry one severe mutation on one *CFTR* gene and a mild mutation on the second *CFTR* gene, and about 12% carry mild mutations on both *CFTR* genes (Claustres et al, 2000). This is in contrast to CF patients, where about 88% of the CF patients carry severe mutations on both *CFTR* genes, and about 11% carry a severe mutation on one *CFTR* gene and a mild mutation on their second *CFTR* gene (Claustres et al, 2000). The most frequent *CFTR* mutation conferring a mild phenotype found in CBAVD patients is the *5T* polymorphism (Chillon et al, 1995). In Caucasians, the *5T* polymorphism is found on about 21% of the *CFTR* genes derived from CBAVD patients, while it is only found on about 5% of the *CFTR* genes derived from control individuals. *5T* is 1 of the alleles found at the polymorphic Tn locus in intron 8 of the *CFTR* gene. A stretch of 5, 7, or 9 thymidine residues is found at this locus. Less efficient splicing will occur when a lower number of thymidines are found, resulting in *CFTR* transcripts that lack exon 9 sequences (Chu et al, 1993). Alternatively, spliced *CFTR* transcripts lacking exon 9 sequences are found in any individual, but the extent varies depending on the alleles present at the Tn locus. *CFTR* transcripts lacking exon 9 sequences result in CFTR proteins that do not mature (Delaney et al, 1993; Strong et al, 1993). When *5T* is found in compound heterozygosity with a severe *CFTR* mutation, or even *5T*, pathology such as CBAVD might be observed. However, not all males who are compound heterozygous for a severe *CFTR* mutation and *5T* develop CBAVD (eg, some fathers of CF children).

2.5 CYSTIC FIBROSIS: TREATMENT

Although there is still no cure for cystic fibrosis (CF), treatment has improved greatly in recent years. The goal of cystic fibrosis treatment is to prevent and control infections of lungs, loosen and remove the thick, sticky mucus from the lungs, prevent blockages in the intestines and provide adequate nutrition. Several treatments are available to ease symptoms and reduce complications (DrugDigest; cystic fibrosis, Mayoclinic; Disease and conditions, cystic fibrosis treatment).

I. Medications

- **Antibiotics.** These drugs are used to treat and prevent lung infections. They may be swallowed in pill form, inhaled in a mist or delivered intravenously.
- **Mucus-thinning drugs.** Drugs that reduce the stickiness of your mucus make it easier to cough up the mucus, which improves lung function.
- **Bronchodilators.** Medications such as albuterol — delivered by an inhaler or a nebulizer — help keep your airways open by relaxing the muscles around your bronchial tubes.

III. **Therapy**

People with cystic fibrosis need a way to physically remove thick mucus from their lungs. This is often done by manually clapping with cupped hands on the front and back of the chest — a procedure that's best performed with the person's head over the edge of the bed so that gravity helps clear the secretions.

VI. Surgical and other procedures

- **Feeding tube.** Cystic fibrosis interferes with digestion, so you cannot absorb nutrients from food very well. The doctor may suggest using a feeding tube to deliver extra nutrition while the patient is sleeping. This tube may be threaded through the nose to the stomach, or surgically implanted.

- **Lung transplant.** The doctor may suggest lung transplantation if the patient has severe breathing problems, life-threatening pulmonary complications or increasing resistance to antibiotics used to treat lung infections. Because both lungs are affected by cystic fibrosis, both need to be replaced. Lung transplantation is a major operation and may lead to serious complications, especially post-surgical infections.

- **Bowel surgery.** If the patient has developed a blockage in his bowel, he may need emergency surgery to remove it — especially if that part of the bowel has died. Intussusceptions, where a section of bowel has folded in on itself, also may require surgical repair.

V. Gene therapy

Gene therapy for cystic fibrosis began in 1990, when scientists successfully corrected faulty cystic fibrosis transmembrane conductance regulator (CFTR) genes. They did this by adding normal copies of the gene to laboratory cell cultures.

In 1993, the first experimental CF gene therapy treatment was given to a patient with cystic fibrosis. Researchers modified a common cold virus to act as a delivery vehicle by carrying normal genes to the CFTR cells in the airways of the lung.

Subsequent studies have tested other methods of gene delivery, such as: fat capsules, synthetic vectors, nose drops, or drizzling cells down a flexible tube to CFTR cells lining the airways of lungs. Researchers are now testing aerosol delivery using nebulizers. (http://www.ornl.gov/sci/techresources/Human_Genome/medicine/genetherapy.shtml. Accessed Sept. 7, 2010).

2.6 CYSTIC FIBROSIS: DIAGNOSIS

Almost 90% of the CF patients are diagnosed in childhood. Some patients are asymptomatic and remain undiagnosed until adolescence or adulthood. A consensus statement was issued by Cystic Fibrosis Foundation in 1998 and diagnosis was suggested by the presence of one or more characteristic clinical features: a history of CF in a sibling, or a positive newborn screening test result and will then be confirmed by laboratory evidence of CFTR dysfunction **2.6.1 Sweat Chloride:**

The standard diagnostic test for Cystic Fibrosis is sweat chloride, the quantitative pilocarpine iontophoresis test (QPIT) to collect sweat and perform a chemical analysis of its chloride content is currently considered to be adequately sensitive and specific test. For reliable results, at least 50 mg or, preferably, 100 mg of sweat must be collected if we use electrical pads, otherwise the minimum acceptable sample is 15 µL in the matter of using electrode sweat chloride. The sweat chloride reference value is less than 40 mmol/L, and a value of more than 60 mmol/L of chloride in the sweat is consistent with a diagnosis of cystic fibrosis. In babies aged 3 months or younger, results of 30-60 mEq/L are considered borderline and require retesting. The sweat test must be performed at least twice in each patient, preferably several weeks apart. The Values of 40-60 mmol/L are considered borderline, and the test must be repeated because these values have been found to be consistent with the diagnosis in some patients with typical features. Repeat a sweat test to confirm positive results. Repeat a sweat test with negative results if clinical features suggestive of cystic fibrosis are present. Some patients with genetically

documented cystic fibrosis and typical symptoms have consistently negative results on sweat tests. A negative result does not rule out the possibilities of CF in these patients.

2.6.2 Nasal Potential Difference (PD) measurements:

Nasal potential difference measurements measure the voltage difference and correlate the movement of sodium across the membrane. The nasal potential difference (NPD) test has been proposed to provide evidence of abnormal function of the cystic fibrosis transmembrane conductance regulator (CFTR), a receptor that forms a chloride ion channel. The test is especially useful for patients who have normal chloride concentrations in sweat tests and in whom 2 gene mutations related to cystic fibrosis have not been detected.

2.6.3 Molecular diagnosis and mutation analysis:

2.6.3.1 Polymerase Chain Reaction (PCR)

For the diagnosis of cystic fibrosis PCR can be used not only for the amplification of target DNA sequence, but also to detect deletion in the gene simply by seeing no product for the target sequence in the agarose gel after the reaction completed, and this can be confirmed by adding suitable internal control when preparing the reaction.

2.6.3.2 Reverse Dot Blot (RDB) by INNO-LiPA CFTR 17+Tn and INNO-LiPA CFTR 19 screening test

Reverse Dot Blot (RDB) or reverse allele specific oligonucleotide (Reverse ASO) hybridization is an important method for genotyping common human mutations (Zhang, 1991, Kawasaki, 1993). RDB was described by Saiki (1989). The method is based on the formation of an amide bond between carboxyl groups present on the membranes and

amino-linkers situated on the 5' end of oligonucleotides. The covalent attachment is via a carbodilmide mediated condensation. The single, 5' end attachment of the oligonucleotides to the membrane surface leaves the probe free to interact with complementary sequences, thus increasing the hybridization efficiency relative to methods where heat or ultraviolet light is used for non-specific fixation.

Using biotinylated PCR products in hybridization reactions along with a non-radioactive chemiluminescent detection system, high efficiency hybridization is obtained as well as a very good signal to noise ratio. The sensitivity, simplicity and reproducibility of this method make it an ideal tool for the diagnosis of infectious and genetic diseases, as well as analysis of mutations in neoplasias, HLA typing and other areas (Zhang, 1991).

INNO-LiPA CFTR 17+Tn and INNO-LiPA CFTR 19 are a line probe assay, for the simultaneous in vitro detection and identification of 17 and 19 human CFTR related mutations and their wild type sequence as well as Congenital Bilateral Absence of the Van Deference (CBAVD)- related Tn polymorphysims from whole blood, buccal brush and blood spot samples.

The INNO-LiPA CFTR test is based on the reverse hybridization principle. Amplified biotinylated DNA material is chemically denaturated, and the separated strands are hybridized with specific oligonucleotide probes immobilized as parallel lines on membrane-based strips. This is followed by a stringent wash step to remove any mismatched amplified material. After the stringent wash, streptavidin conjugated with alkaline phosphatase is added and bound to any biotinylated hybrid previously formed. Incubation with a substrate solution containing a chromogen results in a purple/brown

precipitate. The reaction is stopped by a wash step, and the reactivity pattern of the probed is recorded.

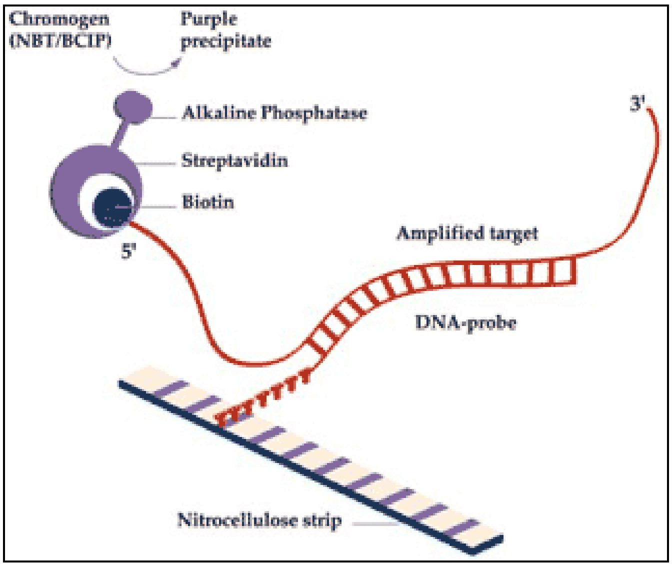


Figure 2.1: INNO-LiPA Principle (www.innogenetics.com)

2.6.3.3 DNA sequencing

In 1988, the first reports of automated DNA sequences were published due to novel techniques of fluorescently label DNA (Smith 1986, Ansorge 1987, Brumbaugh 1988). In the automated Sanger reaction, primers are used that are labeled with four different colored fluorescent tags. PCR reactions, in the presence of the different dideoxy nucleotides. However, the four reaction mixtures are then combined and applied to a single lane of a gel. The color of each fragment is detected using a laser beam and the information is collected by a computer which generates chromatograms showing peaks for each color, from which the template DNA sequence can be determined.

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. PATIENTS

Fifty seven patient samples were included in this study as follows:

Twenty three patients Jordanian were recruited from the Cystic Fibrosis Society and 5 siblings and 29 DNA samples of Jordanians were collected from National Center for Diabetes, Endocrinology and Genetics, used in a previous study (Freihat and El Khateeb, 2007). Reference Preipheral blood was collected from the 23 patients and 5 of their siplings in EDTA tubes and stored at 4C° until DNA extraction. Patients details are shown in table 1.

Table 1: patients Information

Patients	Number	Percentage
Sex: 23		
Male	10	43.5%
Female	13	56.5%
Age:		
0-10	10	43.5%
11-20	9	39.1%
>20	4	17.4%
Parent consanguinity:		
First degree	10	43.5%
Second degree	6	26.1%
No relationship	7	30.4%
Other members in the same family have or died from the disease	5	21.7%

3.2. METHODS

3.2.1. DNA EXTRACTION FROM WHOLE BLOOD

DNA from whole blood was extracted using the manual Wizard Genomic DNA Purification kit (Promega, USA). Around 300 µl of blood was poured in 1.5 ml sterile microcentrifuge tube to which 900 µl of cell lysis solution was added. The tube were mixed by inverted them 5-6 times. The mixture was incubated for 10 minutes at room temperature, and inverted 2-3 times during incubation to lyse the red blood cells. The tubes were centrifuged at (14,000 rpm) for 20 seconds. The supernatant was discarded without disturbing the visible white pellet. Approximately 10-20 µl of residual liquid will remain in the tube. If the pellet appears red, an additional aliquot of cell lysis solution was added after removing the supernatant above the cell pellet, and then repeating the earlier step. The tube is vortexed vigorously until the white blood cells is suspended (10-20 seconds).

Then, 300 µl Nuclei Lysis solution was added to the tube containing the suspended cells. The suspension was pippeted 5-6 times to lyse the white blood cells, where the solution become very viscous. 100 µl protein precipitation solution was added to the nuclear lysate, and the tubes were vortexed vigorously for 10 - 20 seconds then the mixture was centrifuged at 14,000 rpm for 3 minutes. A dark brown protein pellet should be visible. The supernatant was transferred to a clean microcentrifuge tubes containing 300 µl isopropanol each a room-temperature. The tubes were gently mixed until a white thread-like strand of DNA appeared. The tubes were Centrifuged at 14,000 rpm for a minute at room temperature. The supernatant was decanted and one volume of 70% ethanol was added to the palate of DNA. Gently the tubes were mixed and the centrifuge

at 14,000 rpm for one minute. The supernatant was removed carefully. The tubes were inverted on a clean absorbent paper and air-dries the pellet for 10-15 minutes.

Finally, 100 µl DNA rehydration solution was added to each tube to rehydrate the DNA by incubating the solution overnight at room temperature.

3.2.2. DETERMINATION OF THE DNA CONCENTRATION

DNA concentration was determined by using GeneQuant II spectrophotometer using two wavelength 260nm or 280nm, for the quantification and the purity checking . The instrument can measure DNA applying the formula: A_{260} x factor = µg/ml concentration. And the dilution factor for the dsDNA is 25. The purity can be checked by taking the ratio A_{260}/A_{280} which should 1.2 - 1.9 for pure DNA. Pure water was used as blank.

3.2.3.1. MUTATION SCREENING USING INNO-LiPA CFTR 17+ Tn UPDATE AND INNO-LiPA CFTR 19.

Genomic DNA of each CF patient was screened for 36 worldwide most common mutations among the CFTR gene using the INNO-LiPA CFTR 17+Tn Update and INNO-LiPA CFTR 19 diagnostic kits as shown in table 2

3.2.3.1.1. PCR mixture preparation

Two PCR mixtures were prepared, one PCR tube for primer 1 (PS1), and a second one for primer 2 (PS2). Twenty Five µl is the final PCR mixture volume for each tested sample, which includes: 10µl amplification buffer, 5 µl primer solution (PS), 0.5 µl Taq DNA polymerase, 4-5 µl sample DNA , with Deionized water added to bring up the volume to 25 µl. The samples ran in a thermal cycler according to the following program (Table 3.3).

Table 3.2: INNO-LiPA mutations, This table shows the 36 mutations can be detected by INNO-LiPA Kit.

Mutation	Exon	Intron	Mutation	Exon	Intron
Δ508	10		3199del6	17a	
G542X	11		3120+1G>A	16	
N1303K	21		Q552X	11	
W1282X	20		621+1G>T	4	
G551D	11		3849+10kb C>A		19
1717-1G>A		10	2183AA->G	13	
R553X	11		394delTT	3	
CFTRdel2,3	2,3		2789+5G>A		14b
1507del	9		R1162X	19	
711+1G>T		5	3659delC	19	
3272-26A>G		17a	R117H	4	
3905ins T	20		R334W	7	
R560 T	11		R347P	7	
1898+1G>A		12	G85E	3	
S1251N	20		1078delT	7	
I148T	4		A455E	9	
2143delT	13		E60X	3	
2184delA	13		711+5G>A		5
Tn		IVS8			

Table 3.3: PCR program used in INNO-LiPA amplification and conventional PCR.

Functional Step	Temp ^{o}C	Time (min)
1) Primary denaturation	95	5
2) Denaturating of the Template	95	1
3) Annealing of Primers	56	1
4) Elongation of product	72	1
5) Final Elongation	72	10
6) Cooling	4	∞
* Repeat step (2-4) 30 times.		

3.2.3.1.2. Test Procedure

A shaking water path was heated to 47±0.5°C. Hybridization solution and stringent wash solution was prewarmed in the water bath. A required number of test troughs was taken and placed into the tray. Ten µl denaturation solution was pipette into the upper corner of each trough. Then, 10 µl of primer solution 1 (PS1) was added carefully and mixed with the denaturation solution by pipetting up and down. Ten µl of primer solution 2 (PS2) was added to the mixture and mixed by pipetting. The denaturation was allowed for 5 minutes at 20-25 °C. Prewarmed Hybridization solution was mixed and 2 ml was added to the denatured amplified product into each trough. Using forceps, the required number of INNO-LiPA CFTR 17+Tn and INNO-LiPA CFTR 19 strips was taken from the container, one strip for each test and put immediately in the trough. The trough was placed in the water bath and incubated 90 minutes. After Hybridization, the trays were removed from the water bath and held in low angle to aspirate the liquid from the tray with a pipette. Two ml of prewarmed Stringent was added and rinsed by rocking the tray briefly for 20 seconds at room temperature. The wash step was repeated once. Two ml of the prewarmed stringent solution was added and incubated for 30 minutes in the water bath.

During the Stringent step, a concentrated Rinse solution 5x and Conjugate Solution was diluted as follow: Rinse Solution was diluted 1/5 in distilled water (8 ml Rinse Working Solution was prepared for each strip), Conjugate Solution was diluted 1/100 (2 ml Conjugate Working Solution was prepared for each strip).

Trough was removed from the water bath and washed by the 2 ml Rinse Working Solution twice for a minute. Then 2 ml Conjugate Working Solution was added and incubated at room temperature for 30 minutes while agitating the tray on the shaker.

Before 10 minutes of the conjugate period end, the BCIP/NBT Substrate Solution 100x was diluted; it was diluted 1/100 in a substrate buffer; 2 ml of Working Substrate Solution prepared for each strip.

After incubation, each strip was washed twice for a minute with 2 ml Rinse Working Solution, and washed once with 2ml Substrate Buffer. 2 ml of diluted Substrate Working Solution was added to each trough and incubated at room temperatures for 30 minutes while agitating the tray on the shaker. The color development was stopped by washing the strips twice in 2 ml distilled water while agitating the tray on the shaker for at least 3 minutes.

Using the forceps, the strips were removed from the trough and placed on absorbent paper.

3.2.3.2. POLYMERASE CHAIN REACTION (PCR)

Five CFTR exons were analyzed for each patient using this method. The details for each primer sequence and size product and annealing temperature are summarized in Table 3.3.

Deletions in exon 2 and exons 17-18 were detected by conventional PCR and appropriate internal control, and the product was checked on 2% agarose gel at 130V for 1:00 hr.

Table 3.4 : Primers used in this study			
Exon	Primer sequence 5'-3'	Product size (bp)	annealing temp (C°)
Exon 4	4 i-5: CACATATGGTATGACCCTC 4 i-3: TGTACCAGCTCACTACCTA	408 BP	58
Exon 10	10F: 5’-GCAGAGTACCTGAAACAGGA-3’, *10R:* 5’-CATTCACAGTAGCTTACCCA-3’	451 BP	55
Exon 11	11F 5:’-TCAGATTGAGCATACTAAAAG, 11R 5:’-AGCAATAGAGAAATGTCTGTA-3’;	377BP	58
Exon 13	13F 5':TGCTAAAATACGAGACATATTGCA-3 13R5':ATCTGGTACTAAGGACAG-3’	486 BP	57
Exon 19	19F 5’:GCCCGACAAATAACCAAGTGA-3’, 19 R5’-GCTAACACATTGCTTCAGGCT-3	355 BP	58
Exon 2	2F 5’: CATAATTTTCCATATGCCAG-3’ 2 R5’: CACCATACTTGGCTCCTATT-3’	333 Bp	55
Exon 17a	17ae5: TGCAATGTGAAAATGTTTAC 17ae3: CTCTTATAGCTTTTTTACAA	283 BP	55
Exon 17b	17bi5: TTCAAAGAATGGCACCAGTGT 17be3: TGCAGCATTTTATTCATTGA	452	55
Exon 18	18i5: GTAGATGCTGTGATGAACTG 18i3: AGTGGCTATCTATGAGAAGG	390 BP	56

The genomic DNA was extracted as previously mentioned and amplified exponentially by PCR to facilitate analysis. In a standard PCR setup a master mix was prepared. A negative control was used to check out for contamination. The final volume for each reaction tube contain 1µl of dNTPs (Invitrogen, USA), 10µl of 10x PCR buffer, (Invitrogen, USA), 0.3 µl of Taq DNA polymerase (Invitrogen, USA), 1µl of forward and reverse primers (optimal concentration is 10 pmole), 2 µl of 25mmol $MgCl_2$, and

nuclease free water to reach 50 µl final volume. Around 4-5 µl of DNA was added. Then, the samples were run in a thermal cycler.

3.2.3.3 PCR PRODUCT DETECTION BY AGAROSE GEL ELECTROPHORESIS

A 2% agarose mixture was prepared in a glass flask in which a 100 ml of (Tric Boric-acid EDTA) TBE 1x buffer is mixed with 2 gm of agarose powder and heated in a microwave for 3-4 minutes until the agarose melted. The solution was poured in a plastic mould with 24-32 well combs inserted. The gel was allowed to harden for 20-40 minutes at room temperature in a fume hood (30-40 µl of diluted ethedium bromide were also added to the solution). When it became hard, the gel was inserted in a TBE 1x buffer and the comb removed. Around 4-5 µl of EACH PCR product were loaded in the gel along with a 100bp ladder (Promega, USA) to allow the determination of the molecular weight and quantification of DNA. The gel was connected to a power supply (BIO-RAD, USA) with applied voltage of 120v for 20 minutes. Finally, the gel was visualized under UV light or by using a gel documentation system where can be printed.

3.2.3.4 DNA Sequencing

Sequencing was performed for the 44 samples for exons 4,10,11,13 and 19. Around (280) reactions were cleaned up before doing the sequencing. Before and after cleaning, samples were run on 2% agarose gel to confirm the intensity of the product.

3.2.3.5 DNA Purification and cleaning-up

The PCR product was cleaned up using a DNA Purification and clean-up kit (Promega, USA). An appropriate number of Eppendrof tubes and sequencing filters were prepared. An equal volume of Membrane Binding Solution was added to the PCR product and mixed by pippetting and transferred to an Eppendrof tube with attached filter and allowed

to stand for a minute. Then, the tubes were centrifuged at 14,000 rpm for one minute, followed by decantation of the liquid. Then, 700 µl of Washing Membrane Solution was added and centrifuged at 14,000 rpm for 1 minute. Another 500 µl of Washing Membrane Solution was added and centrifuged at 14,000 rpm for 5 minutes, this step was repeated to make sure the elution was free from all the unneeded substances. Then the filter was transferred to a clean Ependrof tube and 50 µl of Nuclease Free Water was added and centrifuged for one minute. Finally, 5 µl of purified DNA is electrophoresed on 2% gel to check the presence and intensity of the product. The samples were sent to Korea (Macrogen, Advancing through Genomics) for sequencing.

4. RESULTS

4.1 Mutation analysis

When we screened the 23 patients (46 alleles) recruited from the Cystic Fibrosis Society, for the 36 mutations (Table 3.1) that are available in INNO-LiPA kits, only four mutations in 5 alleles (10.9%) were identified. These mutations were: ΔF508 in two alleles, N1303K in one allele, R117H in one allele and 2183AA->G in one allele (Figure 4.1).

INNO-LiPA Identification results

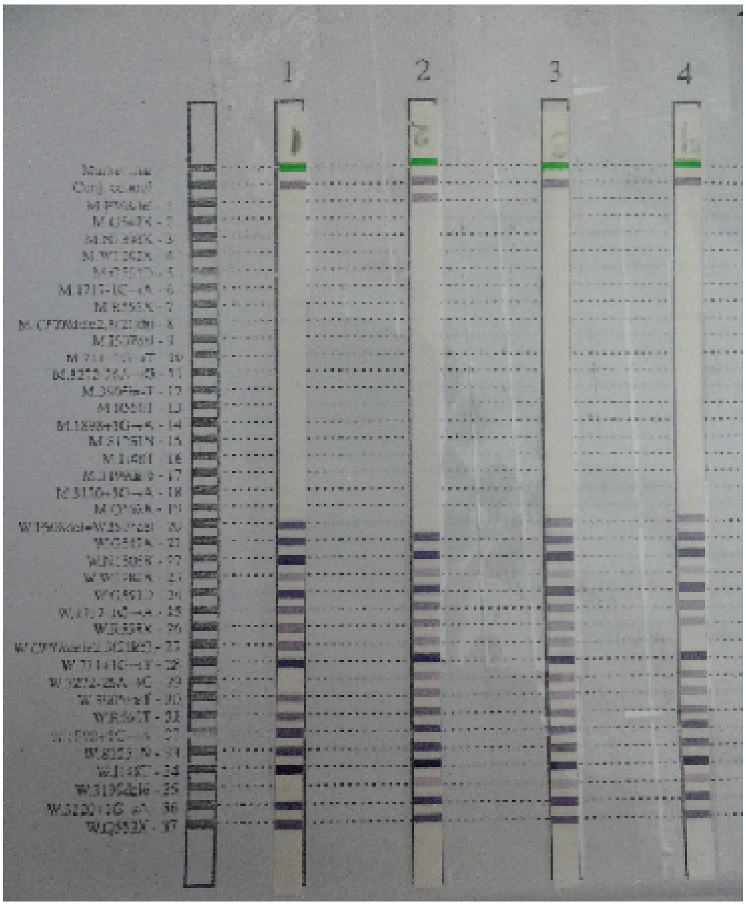


Figure 4.1:
Strip 1: shows negative but also deletions in 3199del6 and 3272-26A>G which is found in exon 17a.
Srtip2: shows a homozygous pattern of Δ508F mutation.
Strip 3: is negative for mutations.

The remaining 41 unidentified alleles were subject for the deletion of whole Exon 2, and exons 17, 18; 6 alleles (13.0%) were found; 2 alleles have exon 2 deletion; 4 alleles have exons 17-18 deletions. Figure (4.2), Figure (4.3), Table (4.3).

Some strips as seen in the INNO-LiPA assay have deletions in some bands despite its absence in wild type (reference test), These deletions were confirmed by conventional PCR and were found to be a deletions in exons 17-18.

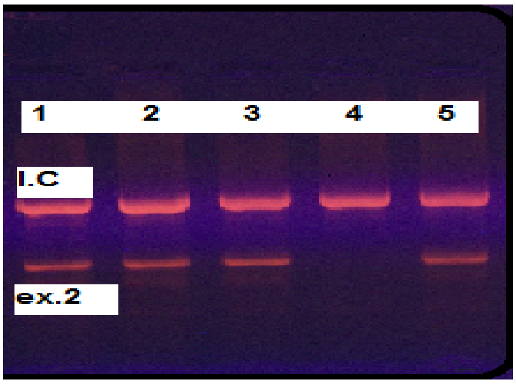
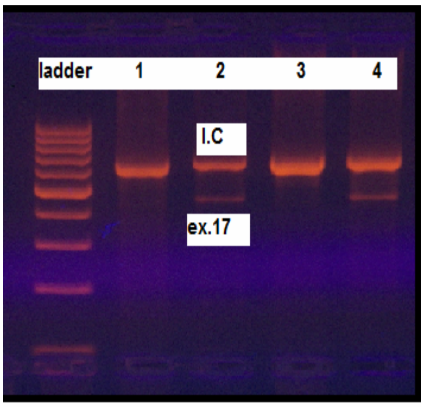
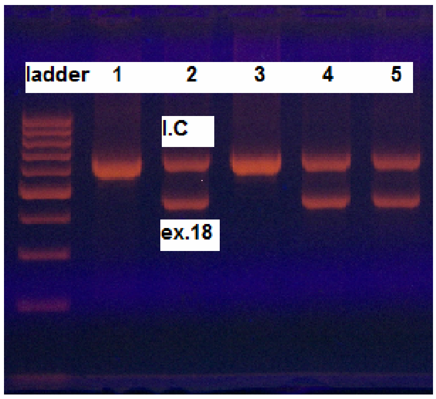


Figure 4.2: deletions of Exon 2 mutation.
Patients 1 , 2, 3and 5 are normal, but patient 4 show homozygous deletion in Exon 2 that present in strip 4 in INNO-LiPA.



4.3A



4.3B

Figure 4.3: Deletions in Exons 17-18

Figure 4.3A: Patient no.2 and 4 are normal; patient no.1 and 3 shows a homozygous deletion in Exon 17.

Figure 4.3B: Patient no.2, 4and 5 are normal; patient no.1 and 3 shows a homozygous deletion in Exon 18.

The remaining unknown alleles along with the 29 DNA samples (taken from NCDEG) were sent for sequencing for the following Exons: 4, 10, 11, and 19. As exon 13 is long (724 bp), only 457 bp were examined by sequencing.

The results were the following (Table 4.1):

Table 4.1: The Sequencing Mutation Results.

Exon	Mutation	Allele (88)	%	Remarks
Exon 4	-	-		Normal.
Exon 10	- Δ508F - 1548elG	2 2	2.3% 2.3%	Known Mutations in Worldwide
Exon 11	- A559T	2	2.3%	Not reported in Middle East.
Exon 19	-	-	-	Normal
Total	3	6	6.9%	

(Wiley-liss, 2002).

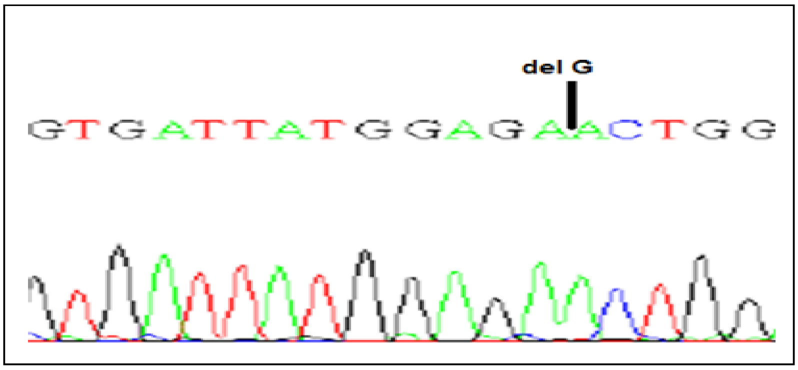


Figure 4.4: 1548delG mutation (Homozygous).

DNA sequencing with a forward primer shows 1548delG mutation.

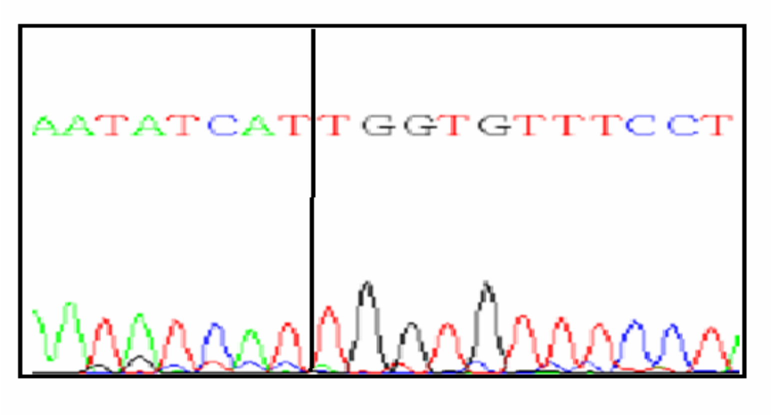


Figure 4.5: Δ508F mutation (Homozygous)

DNA sequencing with a forward primer shows deletion of 3 nucleotides (CTT) that represents Δ508F mutation.

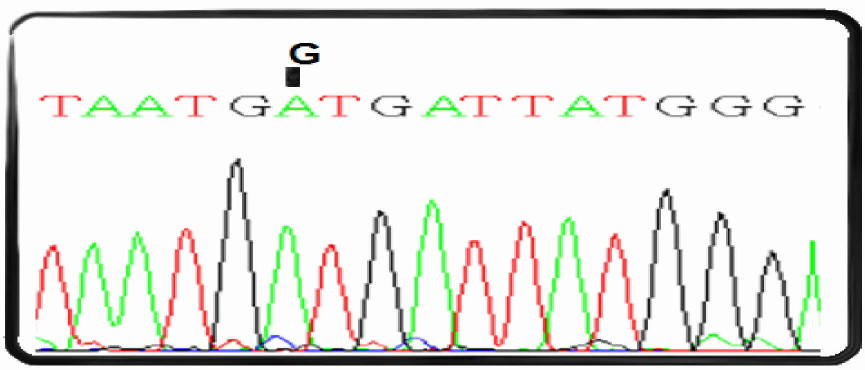


Figure 4.6: polymorphism 1538 G>A (Homozygous)
DNA sequencing with a forward primer shows the polymorphism 1538 G>A where G nucleotide is replaced with A nucleotide.

Sequencing also revealed; 2 novel polymorphisms; one in exon 10 (1538 G>A) and the other one in exon 11 (1744 A>C). (Figure 4.6), (Table 4.3).

Both of these polymorphisms are found in highly variable regions in the exons where a good number of polymorphisms and mutations are found.

The following table shows the overall mutations that were detected in this study:

Table 4.2: List of Mutations Identified in this study

Mutation	Exon/Intron	No. of chromosomes (88 alleles)	%
Δ508	10	4	4.5
N1303K	21	1	1.1
R117H	4	1	1.1
Exon 2 deletion	2	2	2.3
Exon 17-18 Del.	17,18	4	4.5
1548delG	10	2	2.2
A559T	11	2	2.2
2183AA->G	13	2	2.2
Total		18	20.1%

Table 4.3: List of polymorphisms identified in this study

Polymorphism	Exon/Intron	No. of chromosomes (88 alleles)	%
1744 A>C	11	- Heterozygous1744 A>C	2.2%
1538 A>G.	10	- 12 : Heterozygous 1538 G>A - 26 : Homozygous 1538 G>A	13.5% 29.5%

5. DISCUSSION

Cystic Fibrosis (CF) is the most common recessive genetic disease among Caucasians. CF affects approximately 75,000 children and young adults. It occurs in approximately one of every 2500 Caucasians live births (Welsh, 1995). The disease is caused by the alteration in Cystic Fibrosis Transmembrane Regulator (CFTR) gene which functions as a regulator of Cl channel and other channels in epithelial cells (Welsh, 1995). Over 1200 mutations were identified for this gene and reported to the cystic fibrosis mutation consortium data base (genet.sickkids.on.ca). The frequencies of each mutation vary from region to region depending on the geographic and ethnic origin. The most common mutation in CFTR is a deletion of three nucleotides that leads to deletion of one amino acid (phenylalanine) at position 508 and is present in 67% of Caucasians with cystic fibrosis worldwide (Cystic Fibrosis analysis consortium, 1994).

Several reports indicated the presence of CF in different Arab groups where it is generally considered very rare in these populations. Such studies showed that the mutations in Arabs differ from region to region, and that the Δ508 deletion is not a common mutation between Arabs including Jordanians (7.4%), Algerians (20.0%) and Lebanese (35.0%). Furthermore, new mutations have been reported in Arabs that were never identified before in Caucasian CF patients (Desgeorges, 1997; El-Harith, 1997).

This study is designed to reveal the type of mutation in CFTR gene and to be able to establish the frequencies in Jordanian populations. The INNO-LiPA kit (Innogenetics, Belgium) was used as a screening test for the 36 common mutations worldwide. Unidentified mutations were subject to conventional PCR to find out the deletions of

exons 2, 17, 18. The rest of unidentified mutations were screened for by DNA sequencing.

The detection rate by the INNO-LiPA kit was very low, only 4 mutations were identified out 36 (12%). These were: Δ508, N1303K, 2183AA->G and R117H. Despite the low rate, the kit contains only 20 strips with high cost of around 1000 JD.

Sequencing is considered the best strategy for identifying mutations. The detection rate by sequencing in our case was significant where another 3 mutations were identified: homozygous Δ508, 1548delG and heterozygous A559T mutations (samples obtained from NCDEG). Moreover, 2 novel polymorphisms were also identified by sequencing.

Few reports have been published on the incidence of cystic fibrosis among Jordanians in particular and Arab ethnic populations in general. The overall distribution of the CFTR gene mutations among Jordanians differ from those for the United States Caucasians and what have been reported elsewhere in the Middle East (Degeorges, 1997, Frossard, 1994).

In our study, 88 chromosomes from 44 unrelated cystic fibrosis patients were screened for 36 mutations (Table 3.3); 4 mutations were identified: ΔF508, N1303K, R117H and 2183AA->G, followed by the detection for whole exons 2 deletion and for exons 17-18 deletions in-order to reduce the frequency, where 2 alleles showed positive results for exon 2 (2.3%) and 4 alleles for exons 17-18 (4.5%). Sequencing was the last step in the detection strategy, where 3 mutations were identified: (ΔF508, 1548delG and A559T), along with 2 novel polymorphisms which were: (1744 A>C and 1538 A>G).

Despite the fact that the frequency of the ΔF508 mutation in this study was 4.5% which is considered low, it is still, in comparison, the most common mutation worldwide. Our results are in agreement with other studies conducted in the Middle East and North Africa which include: Lebanon 35% (Desgeorges, 1997), Tunisia 50.7% (Messaound, 2005), Turkey 23.5% (Kilinc, 2002) and Algeria 20% (Loumia, 1999).

In Jordan, and in another study performed by Dr Rawashdeh in 2002, 7.4% was the frequency of Cystic Fibrosis.The frequency of ΔF508 is considrably low in comparison to other communities, such as British 80% (Watson, 1990), Italians 55% (Novelli, 1990) and Spanish 50% (Estivill, 1989).

Kambouris (2000), reported 5 common mutations among Arabs: (1548delG, I1234V, ΔF508, 3120+1G>A, H139L) accounting for around 60% of CF alleles in their study. We were unable to confirm their results and only 2 mutations were found: ΔF508 and 1548delG, with low rate of 4.5% and 2.2%, respectively.

The 1548delG mutation that was detected by sequencing in this study was unknown sample from the previous study which was conducted by (Friehat.A and Khateeb.M). It was subjected to INNO-LiPA, SSCP and DGGE, for unspecified reason, it remained unknown. But when it was sent for sequencing, 1548delG was detected. This gives a hint that sequencing is the best mutation detection strategy.

The 1548delG mutation frequency was 2.2% for Jordanian patients which is considered low, but in 1548delG is found in Saudi Arabia with frequency (17%). (El-Harith, 1997).

The A559T mutation was not reported in Middle East before; this mutation was identified in the United States of America in 2.0% of patients of African American origin. This is almost identical to our study (Macek, 1997, Dőrk, 1998, Friedman, 1998).

Screening for common CF mutations that were identified is less than 6.6% of CF alleles in Jordanians, while the total percentage of mutation detected by this study only represents 20.1% which leave 79.9% unknown.

Although no common polymorphisms were detected in this study, two novel polymorphisms were identified, 1744 A>C (exon 11) and 1538 A>G (exon 10). Both were detected by sequencing. 1744 A>C is present in exon 11 and in a high variable region which has a good number of polymorphisms and mutations such as 1738 A>G, 1738 A>T, deletion of AC from 1742 or 1743 C>A or C>G or other polymorphisms. This new polymorphism represents only about 2.2%. of alleles in this study.

The other novel polymorphism detected here is 1538 A>G (Exon 10) which was found in 36 alleles; 12 alleles showed heterozygous pattern (13.5%) and 26 alleles showed homozygous pattern (29.5%) Also, this region is considered as a highly variable area where other mutations and polymorphisms are present such as 1535 C>T, 1537 A>G or a deletion of 10bp at 1540, which implies that regions of the gene can be highly pleomorphic and not easily detected.

Conclusion and Recommendations

In conclusion, the screening for common Caucasian CF mutations identified less than 6.6% of CF alleles in Jordanian CF patients. The commonly used INNO-LiPA kit (Innogenetic, Belgium) screens for 36 mutations. However, the total percentage of mutations detected in this study represents over 20.1%, which still leaves 79.9% of alleles unidentified.

In our study, we only covered 5 exons out of 27, in addition to the deletions in exon 2 and deletions in exons 17-18 along with the common mutations worldwide by screened for by the INNO-LiPA screening test. So, further investigation must be performed on Jordanian patients in order to determine the common mutations in our population. This will facilitate the establishment of a national screening test for cystic fibrosis in the future.

As a result of this study, this can also help in prospective carrier screening tests as well as prenatal screening to guarantee better quality of life for the patients and help reduce the number of affected fetuses in the following pregnancies. When the baby is screened for CF and is confirmed to be affected, the parents should prepare themselves to handle their baby emotionally and medically.

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Consent form for the identification of cystic fibrosis mutations in Jordan

نموذج موافقة المريض على تحديد نوع الطفرات المسببة لمرض التليف الكيسي في الأردن

***هدف هذه الدراسة هو تحديد نوع الطفرات المسببة لمرض التليف الكيسي كرسالة ماجستير في العلوم الطبية المخبرية في الجامعة الأردنية للطلبة سناء خليل الصلاحات.**

الفحص المطلوب هو:

1. المسح عن وجود الطفرة.
2. الكشف عن الشخص الحامل للطفرة.

أنا الموقع ادناه: ----------------------------- أوافق على أخذ العينة المناسبة التي أو من طفلي لإجراء الفحص أو الفحوصات المطلوبة أعلاه و ذلك لتشخيص المرض, علماً بأن الباحث قد شرح لي أسس إجراء هذه الدراسة و الفوائد و المخاطر المتعلقة بها, و قد أجاب على جميع استفساراتي و تفهمت أهمية إجرائها و المحددات لها:

1) مع أن إجراء هذه الدراسة الوراثية كوسيلة لتشخيص المرض إلا أنه في بعض الأحيان لا يعطي الجواب الكافي لتشخيص الحالة الوراثية المعنية لذلك المريض.

2) هذا الفحص خاص بالحالة المرضية المذكورة أعلاه.

3) مع أن تعيين الطفرة أو الحالة المرضية يمكن أن يوفر المعلومات المحددة بدقة, إلا أنه في بعض الأحيان هناك إمكانية حدوث أخطاء منها (أخطاء التشخيص السريري, معلومات خاطئة عن العينة, تلوث العينة و غيرها)ز

4) بعض المواد المستخدمة للتشخيص لا تزال تستعمل في الأبحاث و لهذا السبب يمكن أن تحدث أخطاء أثناء التشخيص, و في بعض الأحيان تكون عملية التشخيص المخبري معقدة مما يستوجب أخذ عينة جديدة من المريض.

5) بعد الانتهاء من إجراء الفحص و ظهور النتيجة (أوافق ---, لا أوافق ---)على حفظ العينة المتبقية لدى المختبر, و لا امانع من استخدامها للبحث العلمي بحيث تزال جميع العلامات الدالة على صاحب العينة.

6) نتائج الفحص ستعامل بسرية تامة و لا تسلم النتيجة إلا للمريض نفسه فقط.

(الباحث)	(المريض أو ولي أمره)
الاسم: -------------------------	الاسم: ---------------------
التوقيع: -----------------------	التوقيع: ---------------------
التاريخ: -----------------------	التاريخ: -------------------

Appendix B:

Reagents supplied by INNO-LiPA CFTR 17+Tn & INNO-LiPA CFTR 19 Update:

Amplification Buffer

Containing all dNTPs and 0.05% NaN_3 as preservative.

Primer Solution Part 1:

Containing biotinylated primers $MgCl_2$ and 0.05% NaN_3 as preservative.

Primer Solution Part 2:

Containing biotinylated primers $MgCl_2$ and 0.05% NaN_3 as preservative.

Denaturation Solution:

Alkaline solution containing EDTA.

Hybridization Solution:

SSC buffer containing 0.5% SDS.

Stringent Wash Solution:

SSC buffer containing 0.1% SDS.

Conjugate 100x:

Streptavidin labeled with alkaline phosphatase in Tris buffer containing protein stabilizers and 0.01% MIT, 0.098% CAA as preservative.

Conjugate Diluent:

Phosphate buffer containing NaCl, Triton®, protein stabilizers and 0.01% MIT, 0.098% CAA as preservative.

Substrate BCIP/NBT 100x:

BCIP and NBT in DMF.

Substrate Buffer:

Tris buffer containing NaCl, $MgCl_2$ and 0.01% MIT, 0.1% CAA as preservative.

Rinse Solution 5x:

Phosphate buffer containing NaCl, Triton®, and 0.05% MIT, 0.5% CAA as preservative.

Appendix C:

Compositions of Buffers and Solutions prepared for the purification of PCR Product:

Membrane Wash Solution;

(After adding Ethanol)

10mM potassium acetate.

80% Ethanol.

16.7µM EDTA (pH 8.0).

TBE 1x

To prepare this solution, add 95% ethanol to the supplied Membrane Wash Solution (concentrated).

Membrane Binding Solution:

4.5 M guanidine isothiocyanate.

0.5M potassium acetate (pH 5.0).

تحديد طفرات التليف الكيسي بين المرضى الأردنيين

إعداد
سناء خليل الصلاحات
المشرف
الأستاذ الدكتور محمد الخطيب
المشرف المشارك
الدكتور سعيد إسماعيل

الملخص

التليف الكيسي هو اختلال وراثي متنحي مهدد للحياة . العامل الوراثي لهذا المرض محمول على الصبغات الجسدية مع اختلاف كبير في العرض السريري يتسم بتغيرات في موروثات CFTR الموجودة على الصبغي السابع الذي يشفر لعنصر مهم في تنسيق حركة الشوارد بين طلائي اغشية الخلية. موروث CFTR يحتوي على أكثر من ألف طفرة مسببة للمرض ومئتي طفرة لا تأثر على وظيفة الموروث .تختلف انتشار هذه الطفرات حسب المنطقة الجغرافية والأصول العرقية.

تهدف هذه الدراسة لتحديد الطفرات الأكثر شيوعا بين المرضى الأردنيين وقياس فاعلية الأطقم التجارية الجاهزة لتشخيص هذا المرض.

أجريت هذه الدراسة على 54 مريض أردني مصاب بالتليف الكيسي.أجري الفحص على عينات جميع المرضى للكشف عن وجود الطفرات الأكثر شيوعا في الجنس القوقازي، وذلك بمسح عينات هؤلاء المرضى ل 36 طفرة شائعة عالميا متوزعة على أقسام المورثة لهذا المرض وهي:(EXSON 2,3,4,7,9,10,11,13,16,19,20,21 and introns 5,12,14b,17a and 19 وذلك باستخدام تفاعل البلمرة المتسلسل , والمتمثلة في الأطقم الجاهزة المستعملة في المملكة وهي INNO_LiPA17+TN والمحدثة و INNO_LiPA. لم تكشف هذه الطريقة إلا عن ثلاث طفرات فقط (F508 and R117H Δ, N1330 K) تشكل أقل من 13 من المرضى.

للكشف عن الطفرات في العينات المتبقية تم استخدام تفاعل البلمرة المتسلسل للكشف عن الحذف الكامل في الإكسونات 2,17_18 . إضافة إلى استخدام DNA sequencing للكشف عن الطفرات الموجودة على الإكسونات 4,10,11,13,19 .

كشفت هذه الدراسة عن وجود ثماني طفرات في المرضى الأردنيين exons . del, (508, G85E,N1303K, del. Exson2 17,18,Q1100P,1548DELG AND 1677delTA)، حيث تشكل ، حيث تشكل 36.2 من المرضى. كانت الطفرة الأكثر شيوعا هي الحذف الكامل

للإكسونات 17,18 بنسبة (11.1) يتبعها الحذف الكمل في إكسون 2 بنسبة (7.4) . ثلاث من هذه الطفرات تكشف لأول مرة في الأردن ؛R117H ,1548delG,N1 303K . بينت هذه الدراسة أن مدى انتشار الطفرة Δ F508الأكثر شيوعا في المجتمعات الغربية لا تشكل أكثر من 6.8 بين مرضانا وهي أقل (على ما نعتقد) نسبة في العالم. عند دراسة الأشكال المتعددة (POLYMORPHISMS) ل EXSON 10 تبين أن نسبة التمايز ل ((1747A>G) كانت 27.7. بالإضافة لهذا التمايز تم الكشف عن طفرتين من الطفرات (1538 A>C)(2.2) كانت غير المسببة للمرض لأول مرة عالميا

الملخص

الخلاصة: بينت الدراسة أن إستعمال الأطقم الجاهزة غير مجدي وأن استخدام الخمس طفرات الأكثر شيوعا (deletion of exons 17-18,del.exon 2 , N1303k,Δ F508, and) يمكن أن تشخص 69 من الحالات . وإن الحاجة قائمة لدراسة وتحديد الطفرات الغير معروفة.